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Anaerobic Degradation of Toluene and Xylene by Aquifer Microorganisms under Sulfate-Reducing Conditions

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Toluene and the three isomers of xylene were completely mineralized to CO_2 and biomass by aquifer-derived microorganisms under strictly anaerobic conditions. The source of the inoculum was gasoline-contaminated sediment from Seal Beach, Calif. Evidence confirming that sulfate was the terminal electron acceptor is presented. Benzene and ethylbenzene were not degraded under the experimental conditions used. Successive transfers of the mixed cultures that were enriched from aquifer sediments retained the ability to degrade toluene and xylenes. Greater than 90% of ^{14}C-labeled toluene or ^{14}C-labeled *o*-xylene was mineralized to $^{14}CO_2$. The doubling time for the culture grown on toluene or *m*-xylene was about 20 days, and the cell yield was about 0.1 to 0.14 g of cells (dry weight) per g of substrate. The accumulation of sulfide in the cultures as a result of sulfate reduction appeared to inhibit degradation of aromatic hydrocarbons.

Soil, sediment, and groundwater are frequently contaminated with petroleum products as a result of leaks in underground storage tanks, improper disposal techniques, and inadvertent spills. Of the many constituents of petroleum, the nonoxygenated, homocyclic aromatic compounds that include benzene, toluene, ethylbenzene, and xylenes (BTEX) are of particular concern because they are confirmed or suspected carcinogens, even at very low concentrations (4). BTEX are relatively water soluble compared with other components of petroleum and thus frequently migrate through groundwater systems to contaminate drinking-water supplies far removed from the actual spill (3, 20). Microorganisms can be (and are being) used to treat water contaminated with BTEX both in the subsurface and in above-ground reactors under both aerobic and anaerobic conditions (3, 12, 15).

The aerobic degradation of BTEX proceeds rapidly and has been studied extensively (8). However, anaerobic conditions often develop in natural ecosystems and in leachate plumes emanating from contaminated sites after oxygen is depleted by aerobic microorganisms (25). The anaerobic transformation of BTEX is not well understood. Indeed, until the mid-1980s, it was generally believed that BTEX were recalcitrant to degradation under anaerobic conditions. The first documented report for benzene and toluene degradation under anaerobic conditions appeared in 1980, concerning studies of biodegradation in microcosms containing samples from the site of the Amoco Cadiz oil spill (23). In 1984 and 1985, field evidence for anaerobic degradation of BTEX in contaminated leachate plumes and aquifers was reported (13, 20). Laboratory studies demonstrated that many different electron acceptors could replace oxygen in the anaerobic degradation of BTEX compounds. Certain BTEX have been shown to be degraded under denitrifying conditions (5, 7, 13, 14, 18, 26), under methanogenic conditions in microcosms (9, 23–25), and by iron-reducing organisms (16, 17). However, the existence of microbial communities capable of degrading BTEX with sulfate as the electron acceptor has not yet been proven. Haag et al. (11) suggested that sulfate-reducing bacteria were involved in the biodegradation of BTEX, although definitive proof to this effect was not presented. In this article, we provide conclusive evidence linking toluene and xylene biodegradation to sulfate reduction. In a companion article, Beller et al. (1) also show a link between toluene degradation and sulfate reduction in enrichment cultures derived from a different sediment source (Patuxent River, Md.). These two independent studies confirm that sulfate can be used as the terminal electron acceptor for BTEX degradation and that such activity may exist at many contaminated sites where sulfate is present.

(Preliminary results from this study were presented at the International Symposium on *In Situ* and On-Site Bioreclamation, 12 to 21 March 1991, San Diego, Calif. [6].)

MATERIALS AND METHODS

Aquifer material. Samples from a gasoline-contaminated sandy silt (Seal Beach, California) were taken on 12 May 1987, using a steam-cleaned 0.76-m-diameter bucket auger at a depth of 0.25 m above the water table (11). The sediment samples were stored at 4°C in 20-liter plastic containers flushed with N_2 and sealed with air-tight snap lids.

Medium. A medium designed to support sulfate-reducing bacteria was prepared that had the following constituents per liter of deionized water: 10 ml of phosphate buffer (27.2 g of KH_2PO_4 and 34.8 g of K_2HPO_4 per liter), 10 ml of salt solution (53.5 g of NH_4Cl, 7.0 g of $CaCl_2 \cdot 6H_2O$, 2.0 g of $FeCl_2 \cdot 4H_2O$ per liter), 2 ml of trace mineral solution [0.3 g of H_3BO_3, 0.1 g of $ZnCl_2$, 0.75 g of $NiCl_2 \cdot 6H_2O$, 1.0 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $CuCl_2 \cdot 2H_2O$, 1.5 g of $CoCl_2 \cdot 6H_2O$, 0.02 g of Na_2SeO_3, 0.1 g of $Al_2(SO_4)_3 \cdot 16H_2O$, and 1 ml of H_2SO_4 per liter], 2 ml of $MgSO_4 \cdot 7H_2O$ solution (62.5 g/liter), 1 ml of redox indicator stock solution (1 g of resazurin per liter), 10 ml of saturated bicarbonate solution (260 g of $NaHCO_3$ per liter), 10 ml of filter-sterilized vitamin stock solution (0.02 g of biotin, 0.02 g of folic acid, 0.1 g of pyridoxine hydrochloride, 0.05 g of riboflavin, 0.05 g of thiamine, 0.05 g of nicotinic acid, 0.05 g of pantothenic acid, 0.05 g of *p*-aminobenzoate [PABA], 0.05 g of cyanocobalamin, and 0.05 g of thioctic acid per liter), and 10 ml of an

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amorphous ferrous sulfide solution [39.2 g of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and 24.0 g of $Na_2S \cdot 9H_2O$, which had been washed three times with deionized water to remove free sulfide, per liter]. The initial sulfate concentration was 25 mM (Na_2SO_4). Sulfate was added to the medium prior to autoclaving. The vitamins, bicarbonate, and ferrous sulfide were added from sterile stock solutions after autoclaving and cooling the medium and while gassing the medium with N_2-CO_2 (80%:20%). The pH of the medium was usually between 6.8 and 7.2. In the experiments designed to test the effect of pH on degradation, the pH of the medium was adjusted to 6 with 1 N HCl or to 8 with 1 N NaOH.

Chemicals. Chemicals were purchased from Sigma (St. Louis, Mo.) or Aldrich (Milwaukee, Wis.) and were greater than 99.9% pure. [*methyl*-^{14}C]toluene, [*ring*-^{14}C]toluene, and *o*-[*methyl*-^{14}C]xylene were also purchased from Sigma and had specific activities of 4.9, 9.5, and 10.9 mCi/mmol, respectively.

Microcosms. Microcosms were prepared in 250-ml (ca. 8-oz) screw-cap bottles and sealed with Mininert valves (Alltech Associates, Inc., Deerfield, Ill.). The bottles and caps were acid washed, sterilized, and brought into an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) before use. After the glassware had been incubated in the glove box for at least 1 day to remove all traces of adsorbed oxygen, 100 g of aquifer material was transferred into each 250-ml bottle, followed by 100 ml of medium. The bottles were then sealed with Mininert valves. In total, eight microcosms were established: two were autoclaved sterile controls, two were amended with 2-bromoethanesulfonic acid (BESA) at a concentration of 1 mM to inhibit methanogens (10), and the remaining four bottles had no special treatment. The sediment in sterile control bottles was autoclaved for 20 min at 121°C on 3 consecutive days before medium was added to these bottles. All of the bottles were spiked with a stock substrate solution containing a mixture of benzene, toluene, ethylbenzene, *p*-xylene, and *o*-xylene to give an initial concentration of each compound of approximately 5 mg/liter (50 μM). As degradation occurred, additional single substrates were added by injecting 1.5 to 3.0 μl of the pure compound with a 10-μl syringe. Sulfate was also replenished as needed. Periodically, 20 to 50 ml of liquid was removed from the bottles and replenished with fresh sterile medium. All manipulations were performed in an anaerobic glove box (atmospheric composition: 85% N_2, 10% CO_2, 5% H_2). The bottles were incubated anaerobically at 20°C in the dark.

Enrichment cultures. Primary enrichment cultures were prepared by transferring sediment (10 g) and liquid (30 ml) from active microcosms to clean, autoclaved 250-ml bottles. These bottles were then filled with 170 ml of sulfate-reducing medium, gassed with N_2-CO_2 (80%:20%), and spiked with the appropriate substrates (as pure compounds) at concentrations between 8 and 20 mg/liter (80 to 200 μM). Further enrichments were prepared by transferring only the liquid portion of primary enrichment cultures into fresh medium (10 to 30% inoculum) in 250-ml bottles or in smaller 40-ml screw-cap vials (also sealed with Mininert valves). Enrichment cultures that no longer contained aquifer solids were used for most experiments. Some enrichment cultures were spiked with [^{14}C]toluene (both *methyl* and *ring* labeled) or *o*-[*methyl*-^{14}C]xylene (approx. 4,000 dpm/ml) to determine the final products of degradation.

Analytical procedures. The concentration of BTEX was measured by withdrawing 300 μl of headspace from sample bottles with a 500-μl gas-tight syringe and injecting the headspace into a Carlo Erba Fractovap 2900 Series gas chromatograph (GC; Carlo Erba Strumentazione, Milan, Italy) equipped with a photoionization detector (PID; HNU Systems Inc., model PI-52-02; 10-eV lamp). The operating conditions for the GC/PID (DB-624 fused silica megabore capillary column, 30 by 0.53 mm inside diameter; J & W Scientific, Folsom, Calif.) were an injection port temperature of 240°C, a detector temperature of 250°C, helium carrier gas at a column head pressure of 0.7 kg/cm^2, helium make-up gas at a flow of 30 ml/min, an isothermal temperature of 90°C, and a splitless injection (split closed for 30 s). Sulfate was analyzed on a Dionex Series 4000i ion chromatograph (IC) with an electrochemical conductivity detector. The eluant for the IC was 0.75 mM sodium bicarbonate–2.2 mM sodium carbonate (2 ml/min), and the regenerant was 0.025 N sulfuric acid. The data from the GC/PID and IC were collected and processed with the Nelson Analytical Inc. 3000 Series Chromatography Data System. Both the GC and the IC were calibrated with external standards.

^{14}C activity was determined on a Tricarb model 4530 scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Three separate 1-ml liquid samples were counted for each analysis to determine the ^{14}C activity in the volatile, nonvolatile, and CO_2 fractions as described by Grbić-Galić and Vogel (9). Dissolved sulfide concentration (H_2S, HS^-, and S^{2-}) was determined by the spectrophotometric methylene blue method (2). Cell counts were determined in a 10-μl sample spread over a 1-cm^2 area on a microscope slide. The samples were heat-fixed and stained with acridine orange (0.01%) for 2 min and then washed with water. The cells were observed under oil immersion in an epifluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan), equipped with an Olympus FLPL 100× objective lens and a 10× ocular lens. Sixteen to 20 fields per sample were counted, and the average cell count per field was used to calculate the total cell count, given that the area of the field was 1.7×10^{-3} mm^2.

RESULTS

Microcosms. In all of the microcosms (except sterile controls) that were initially fed a mixture of benzene, toluene, ethylbenzene, *o*-xylene, and *p*-xylene (initial concentration of each compound, approximately 5 mg/liter), toluene was the first compound to be degraded. Toluene, *p*-xylene, and finally *o*-xylene were more than 80% degraded by days 40, 72, and 104, respectively. Neither benzene nor ethylbenzene was degraded after 270 days of incubation. None of the compounds disappeared in autoclaved controls (Fig. 1). After this initial adaptation period, toluene, *p*-xylene, and *o*-xylene were refed as needed, and degradation proceeded without a lag.

Dependence on sulfate. Some preliminary observations suggested that sulfate reducers may have been involved in the disappearance of toluene and xylenes. Methane was not detected in the headspace of any microcosm. The resazurin redox indicator remained colorless throughout the experiments, indicating anaerobiosis. The sulfate concentration decreased and a black precipitate formed only in active microcosms, presumably as a result of hydrogen sulfide formation and FeS precipitation. To confirm a link between toluene degradation and sulfate reduction, we monitored toluene and sulfate concentrations during periods when sulfate became depleted. Figure 2 demonstrates the dependence of toluene degradation on the presence of sulfate in a microcosm. The same effects were observed for *p*- and

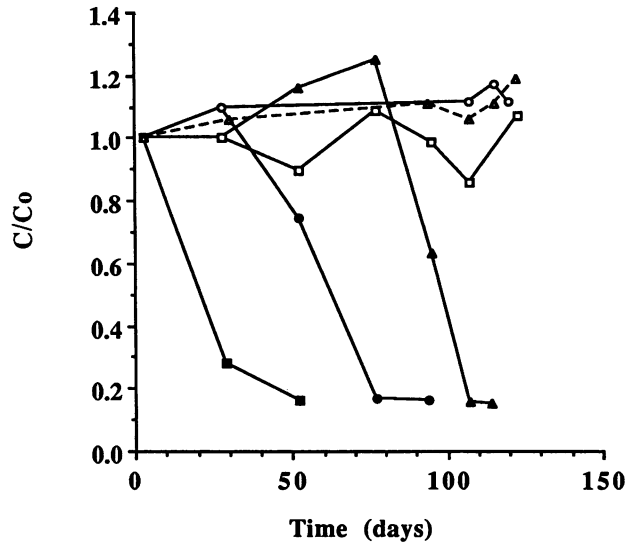

FIG. 1. Degradation of toluene (■), *p*-xylene (●), and *o*-xylene (▲) in unacclimated microcosms. The vertical axis is concentration normalized by dividing the concentration at a given time by the initial concentration of that substrate (C/C_0). The results for active microcosms are the mean of six replicates. The results for the sterile control (--△--) are the mean of two replicates for all five hydrocarbons, including benzene (□) and ethylbenzene (○). The concentrations of toluene, *p*-xylene, and *o*-xylene never decrease to zero because of desorption from the sediments.

o-xylene degradation (data not shown). Degradation proceeds as long as sulfate is present, ceases when sulfate becomes depleted, and resumes upon addition of sulfate. Molybdate (2 mM) completely inhibited toluene degradation, whereas BESA (1 mM) had no effect on the rate of degradation (Fig. 3).

Enrichment cultures. Primary enrichment cultures inoculated with both liquid and a small amount of solid material from active microcosms into defined mineral medium retained activity towards toluene, *p*-xylene, and *o*-xylene. Enrichment cultures also degraded *m*-xylene without a lag (this substrate was not added to microcosms in initial studies

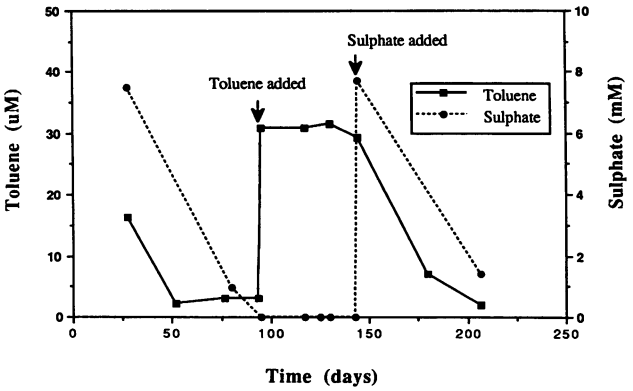

FIG. 2. Effect of sulfate on toluene degradation. Degradation proceeds as long as sulfate is present, ceases when sulfate becomes depleted, and resumes upon addition of sulfate. The data shown were taken from one microcosm only, although the trends are representative of all six active microcosms, and the same effects were observed for *p*- and *o*-xylene.

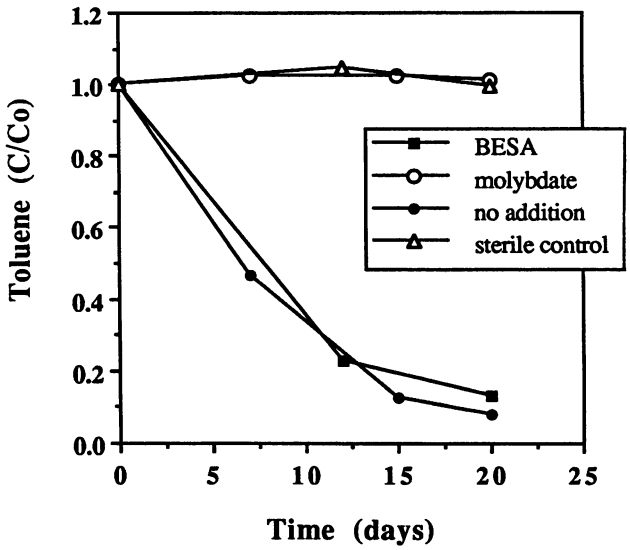

FIG. 3. Effect of BESA (1 mM) and sodium molybdate (2 mM) on toluene degradation in enrichment cultures. Positive controls received neither BESA nor molybdate. The results are the mean of two replicates.

because it coelutes with *p*-xylene on the GC). Subsequently, only the liquid portion of enrichment cultures was transferred, and the activity was retained. These suspended cultures were used for subsequent experiments because enrichment cultures containing no aquifer sediment are needed for mass balance estimations to minimize complications due to sorption and unknown carbon sources and electron acceptors present in the sediment. The measured amounts of sulfate consumed and sulfide produced per mole of toluene or *m*-xylene degraded in enrichment cultures containing no sediment are shown in Table 1, in comparison to the theoretical stoichiometric ratios.

The overall theoretical stoichiometric and energetic equations for toluene and *m*-xylene biodegradation under sulfate-reducing conditions were developed following the method described by McCarty (19), assuming an efficiency of energy transfer of 60%. These equations are reported in Table 2. This method is based on the assumption that a correlation exists between the free energy of reaction and cell yield. On this premise, we calculated that 4.14 mol of sulfate are required per mol of toluene degraded and 4.83 mol of sulfate per mol of xylene degraded and that the maximum cell yield for cultures growing on toluene or xylene under sulfate-reducing conditions would be about 0.2 g of cells (dry

TABLE 1. Measured and theoretical moles of sulfate consumed and sulfide produced per mole of toluene or *m*-xylene consumed[a]

Substrate	Sulfate consumed	Sulfide produced
Toluene		
Measured	4.5 ± 0.3	1.6 ± 0.7
Theoretical	4.14	4.14
m-Xylene		
Measured	4.9 ± 0.4	4.1 ± 1.0
Theoretical	4.83	4.83

[a] Measured values are mean ± standard deviation of three to six replicates.

TABLE 2. Stoichiometry and energetics of toluene and xylene oxidation under sulfate-reducing conditions

Compound	Overall stoichiometry[a]	Energy[b]	
		Equation	$\Delta G^{0'}$ (kcal/mol)
Toluene	$C_7H_8 + 4.14\ SO_4^{2-} + 0.144\ NH_4^+ + 2.568\ H_2O \rightarrow 6.28\ HCO_3^- + 2.07\ HS^- + 2.07\ H_2S + 0.144\ C_5H_7O_2N + 0.214\ H^+$	$C_7H_8 + 4.5\ SO_4^{2-} + 3\ H_2O \rightarrow 7\ HCO_3^- + 2.25\ HS^- + 2.25\ H_2S + 0.25\ H^+$	−54.7
Xylene	$C_8H_{10} + 4.83\ SO_4^{2-} + 0.168\ NH_4^+ + 2.496\ H_2O \rightarrow 7.16\ HCO_3^- + 2.415\ HS^- + 2.415\ H_2S + 0.168\ C_5H_7O_2N + 0.083\ H^+$	$C_8H_{10} + 5.25\ SO_4^{2-} + 3\ H_2O \rightarrow 8\ HCO_3^- + 2.625\ HS^- + 2.625\ H_2S + 0.125\ H^+$	−63.8

[a] Including cell ($C_5H_7O_2N$) formation.

[b] The data for computing free-energy changes were taken from McCarty (19) and Thauer et al. (21). For comparison, the $\Delta G^{0'}$ for the aerobic oxidation of toluene is −910 kcal/mol and of xylene is −1,060 kcal/mol.

weight) per g of substrate. The standard free-energy changes ($\Delta G^{0'}$) for the oxidation of toluene and xylene under sulfate-reducing conditions are also given in Table 2. The amount of energy available from this reaction is about 17 times less than is available for toluene or xylene oxidation under aerobic conditions.

The rates of degradation ranged from 0.1 to 1.5 mg liter^{-1} day^{-1}, depending on the substrate mixture, substrate concentration, and environmental conditions. Typically, toluene and *m*-xylene were degraded more rapidly than *p*- and *o*-xylene. The degradation of toluene, *p*-xylene, and *o*-xylene in enrichment cultures fed a mixture of these three compounds is shown in Fig. 4. Toluene is always the preferred substrate, and there appears to be a sequential degradation of the compounds, with *o*-xylene being degraded last. In the presence of more easily degradable substrates, such as lactate, glucose, and yeast extract, the degradation of toluene and xylenes ceased completely until all preferred substrates were consumed. At total concentrations of toluene and xylenes above about 30 mg/liter (300 μM), the rates of degradation began to decrease. For example, at 40 mg/liter (400 μM), the rate of *m*-xylene degradation was 60% of the rate at 20 mg/liter; at 60 mg/liter (600 μM), no degradation was observed. The optimum pH for degradation was found to be near 7.0 for both toluene and xylenes. At pH 6.0, the rate of toluene degradation was 80% of the rate at pH 7.0, and at pH 8.0 the rate of toluene degradation was 60% of the rate at pH 7.0. At pH 6.0, the rate of *p*- and *o*-xylene degradation was 60% of the rate at pH 7.0, and at pH 8.0, the rate of *p*- and *o*-xylene degradation was 30% of the rate at pH 7.0. Free sulfide strongly inhibited degradation. The addition of 1 mM Na_2S decreased the rates of toluene and xylene degradation by half. The rates of degradation would increase again if the cultures were flushed with N_2-CO_2 and replenished with fresh medium.

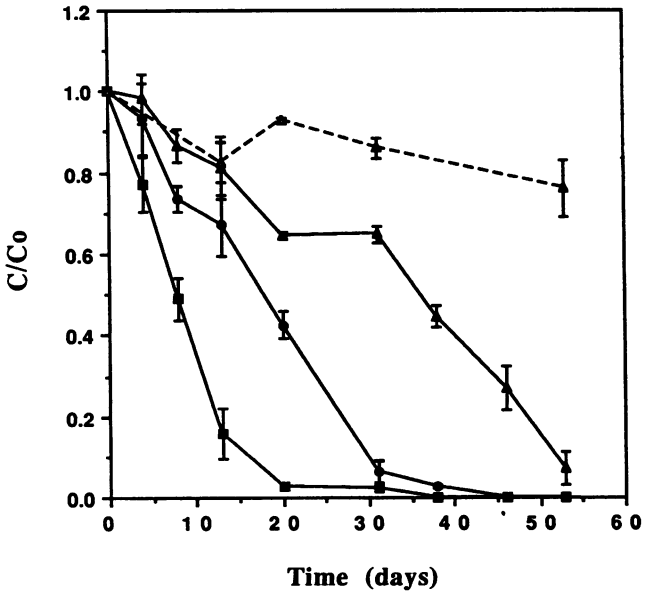

FIG. 4. Sequential degradation of toluene (■), *p*-xylene (●), and *o*-xylene (▲) by a sulfate-reducing mixed culture. In this experiment, 30-ml enrichment cultures in 40-ml vials were fed toluene, *p*-xylene, and *o*-xylene simultaneously at an initial concentration of each compound ranging from 8 to 12 mg/liter (80 to 120 μM). The results for active enrichment cultures are the mean of three replicates. The results for the sterile control (--△--) are the mean of two replicates for all three substrates. Error bars are ± standard deviation.

To determine the ultimate fate of the carbon in toluene and xylenes, some cultures were spiked with ^{14}C-labeled substrates (either *ring*-labeled toluene, *methyl*-labeled toluene, or *methyl*-labeled *o*-xylene). From 95 to 100% of the initial volatile activity was recovered as $^{14}CO_2$ in all cases, indicating complete mineralization of the substrates to CO_2, with only a very small portion of the carbon being assimilated into cells (Fig. 5). To further substantiate these findings, we monitored cell counts while the cultures were growing on toluene or *m*-xylene alone. These cultures exhibited a very long doubling time and a small yield (Table 3), in agreement with the results of the ^{14}C studies. Under epifluorescence microscopy, several cell shapes were apparent. Fat, rod-shaped cells, about 1.3 by 2.8 μm, that often formed chains 2 to 20 cells in length, and long, thin, filamentous organisms about 0.2 μm in diameter were dominant. All cells appeared in close association with solid particles and with each other. Total cell counts ranged from 1×10^6 to 5×10^7 cells per ml.

DISCUSSION

We have provided evidence for the complete mineralization of toluene and xylenes by an aquifer-derived mixed culture utilizing sulfate as the terminal electron acceptor. Toluene and xylene degradation ceased when sulfate became depleted and resumed upon addition of sulfate. Molybdate, an analog of sulfate and an inhibitor of sulfate reducers, completely inhibited the degradation of toluene, while BESA, a potent inhibitor of methanogenesis, had no effect on degradation, further confirming the role of sulfate reducers in the degradation of toluene and xylenes. The Seal Beach aquifer material used in this study was a good

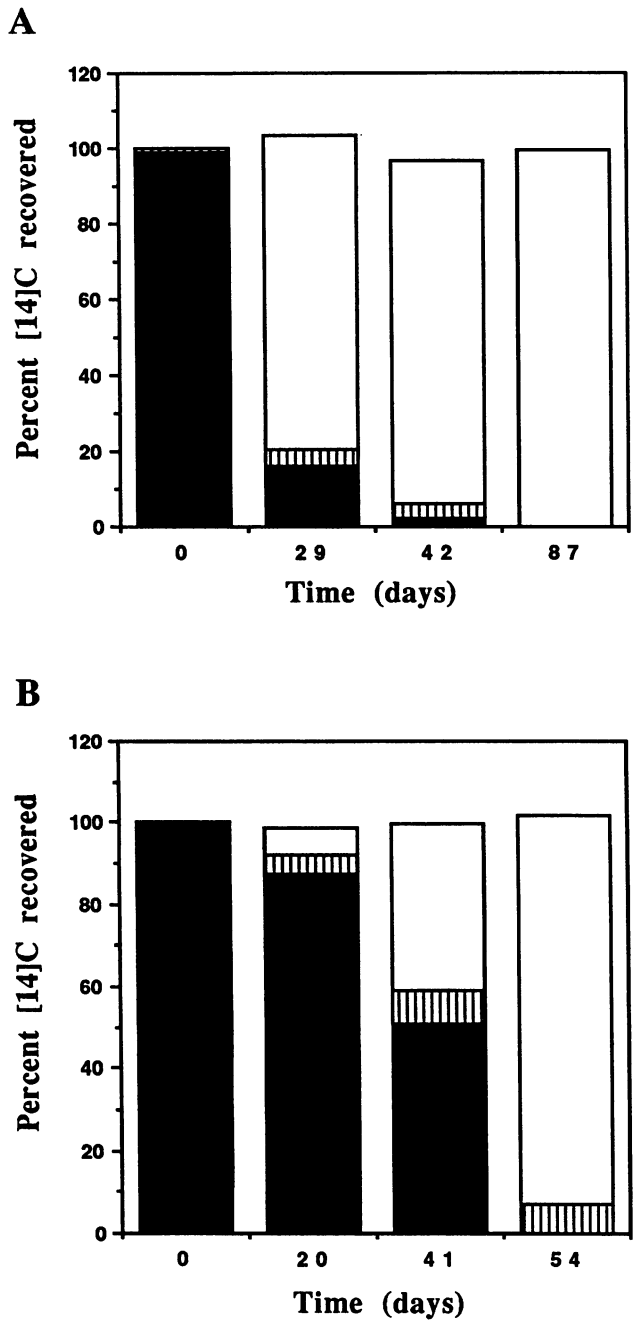

FIG. 5. Fate of ^{14}C-labeled substrates in a sulfate-reducing mixed culture. (A) Means from two cultures incubated with *methyl*-labeled toluene. Similar results were obtained with cultures incubated with *ring*-labeled toluene. (B) Means from two cultures incubated with *methyl*-labeled *o*-xylene. The nonvolatile fraction includes biomass and nonvolatile intermediates. No change in the label distribution was observed in sterile controls. Open bars, carbon dioxide; hatched bars, nonvolatile fraction; solid bars, volatile fraction (toluene [A] or *o*-xylene [B]).

candidate for finding such a community, since the site had been exposed to gasoline and contained relatively high concentrations of sulfate because of its proximity to an intertidal marsh (11). The relatively short adaptation times observed in this study may be due to preexposure of the sediment in situ to these same compounds in an environment where sulfate reduction was occurring naturally. A field study at this site is under way to demonstrate these processes in situ. Benzene and ethylbenzene were not degraded under the experimental conditions used. The recalcitrance of benzene to anaerobic degradation is a recurring finding in recent studies (7, 12, 14). This is unfortunate, since benzene is the most toxic BTEX and a proven human carcinogen and therefore the most crucial compound to eliminate from contaminated sites.

TABLE 3. Cell yield and doubling time for a sulfate-reducing mixed culture growing on toluene and *m*-xylene[a]

Growth substrate	Yield[b] (g of cells/ g of substrate)	Doubling time (days)
Toluene	0.10 ± 0.02	22 ± 4.4
m-Xylene	0.14 ± 0.03	20 ± 4.0

[a] Values are mean ± standard deviation of two or three replicates.
[b] The calculated theoretical maximum cell yield for growth on toluene or *m*-xylene under sulfate-reducing conditions is about 0.2 g of cells (dry weight) per g of substrate.

In microcosms and in primary enrichment cultures that still contained a small fraction of the original sediment, more sulfate was consumed than is theoretically required to degrade the amount of toluene and xylenes added (Fig. 2). We attributed the excess sulfate demand to the presence of organic material on the sediment. These experiments were repeated in enrichment cultures which did not contain sediment, and again we found 30 to 40% more sulfate reduction than theoretically predicted. This discrepancy is explained by the observation that, over the long incubation times of these experiments (several months), H_2 from the glove box atmosphere diffused through the Mininert caps and was utilized by sulfate reducers. The difference in the amount of sulfate consumed between parallel enrichment cultures in the presence and absence of toluene or *m*-xylene yielded stoichiometric coefficients that more closely match the theoretical values (Table 1).

The production of H_2S in microcosms and enrichment cultures was suspected because of the appearance of a black precipitate and from the characteristic smell of this compound. The production of H_2S was confirmed analytically by measuring an increase in dissolved sulfide species during degradation of toluene and xylenes. The method used reportedly measures only soluble sulfides (2), although it involves adding the sample to a mixed diamine reagent that is very acidic, and therefore perhaps some of the acid-soluble sulfide in precipitates such as FeS was also measured. Because of this uncertainty, we believe that the values reported for sulfide production in Table 1 are not reliable enough to be used in a sulfur mass balance, but serve only to confirm qualitatively that sulfide is indeed being produced.

The complete mineralization of toluene and *o*-xylene to carbon dioxide was confirmed by using ^{14}C-labeled substrates. These experiments also demonstrated that very little carbon was being assimilated into biomass. In the experiment shown in Fig. 5A, all of the label was eventually recovered as $^{14}CO_2$, with no residual nonvolatile fraction (day 87). This may indicate that some of the heterotrophs in the community metabolized dead cells from the organisms that degraded the substrates of interest, causing the release of cell carbon as CO_2. Many different bacterial shapes were visible under the microscope. The bacteria appeared in close association with each other and with solid particles, reveal-

ing a potentially very complex community structure able to live off an electron donor/acceptor system that offers little energy.

The growth conditions for the cultures were probably not optimal, as suggested by the extremely long doubling times, the low cell yields, and the variable rates of degradation. The measured cell yields were about half of the theoretical maximum cell yield computed from thermodynamics. We suspect that free sulfide accumulating in the medium as the cultures grew was toxic to or inhibited some of the bacteria in the cultures because of the following observations: (i) the addition of 1 mM Na_2S strongly inhibited degradation, and the addition of 5 mM Na_2S almost completely shut down degradation; (ii) the rate of degradation of toluene and xylene decreased as these substrates were consumed (the degradation of 250 μM toluene would result in the production of 1 mM sulfide, which should strongly inhibit degradation); (iii) flushing cultures with N_2-CO_2, replacing some culture supernatant with fresh medium, or adding 2 mM $FeSO_4$ to remove free sulfide from solution all resulted in increases in the rate of degradation; and (iv) in the original microcosms that contain 100 g of sediment, there is little evidence of sulfide toxicity, presumably because all the sulfide produced can be precipitated out of solution by the minerals in the sediment. Efforts are under way to determine the optimum culturing method to adequately remove the sulfide formed in batch cultures such as these.

An understanding of the factors that influence the anaerobic biodegradation of priority pollutants such as BTEX is necessary to effectively manage and remediate contaminated sites. In this study, we have provided evidence for the existence of microbial communities capable of degrading toluene and xylenes under sulfate-reducing conditions. It is highly likely that this process was occurring naturally at the contaminated Seal Beach site, given that toluene degradation in microcosms began with virtually no adaptation lag (Fig. 1). The enriched mixed cultures described herein showed definite substrate preferences (e.g., toluene over xylenes; lactate, glucose, or yeast extract over toluene) and specificity (benzene and ethylbenzene were not transformed). Typically, mixtures of compounds are present at contaminated sites; therefore, the degradation of contaminants such as BTEX in the field may be prevented not because of the lack of appropriate organisms or enzymes, but because of the presence of other more readily degraded substrates. The biochemical mechanisms for degradation and the role of these bacteria in natural systems remain to be fully elucidated. The mechanism of reaction in these anaerobic systems is especially intriguing given that very little energy is available from the reaction.

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